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Promotional Effects of CO_2 Laser on DMBA-Induced Hamster Buccal Pouch Carcinogenesis as Shown by Immunohistochemistry of the Placental Form of Glutathione S-Transferase

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Background and Objective: **The purpose of the present study was to investigate the kinetics of the expression of the placental form of glutathione S-transferase (GST-P), a useful marker of premalignant lesions, and cell proliferation after CO_2 laser surgery on the carcinogen-initiated epithelium.**
Study Design/Materials and Methods: **CO_2 laser incisions were made on buccal pouch epithelium of 36 hamsters after initiation by 9,10-dimethyl-1,2-benzanthracene (DMBA) (group 1, G1), and scalpel incisions were similarly made on 33 animals (group 2, G2). Twenty animals not treated further after initiation were used as DMBA-treated controls. Incidence of malignant transformation, expression of GST-P, and cell proliferation were examined.**
Results: **The incidence of malignant transformation in G1 and G2 increased significantly (G1: $P < 0.001$; G2: $P < 0.05$) compared with that in DMBA-treated controls. GST-P expression of hyperplasia in G1 and G2 decreased significantly ($P < 0.001$) compared with that in DMBA-treated controls. In hyperplasia, cell proliferation of the GST-P-negative area was significantly ($P < 0.001$) higher than that of the GST-P-positive area.**
Conclusion: **The incisions, particularly by the CO_2 laser, on the initiated areas made expression of GST-P decrease and cell proliferation increase in the GST-P-negative areas. These incisions may serve to promote malignant transformation. Lasers Surg. Med. 24:360–367, 1999.** **© 1999 Wiley-Liss, Inc.**

Key words: cell proliferation; CO_2 laser surgery; 5-bromodeoxyuridine; oral carcinogenesis

INTRODUCTION

The CO_2 laser has been used widely as a surgical tool for treatment of different diseases in the oral and maxillofacial region because it has many advantages, such as improved hemostasis, decreased postoperative pain, and edema [1,2]. A premalignant lesion is one of the most important diseases as an indication of CO_2 laser surgery. Oral leukoplakia, defined as a white patch or plaque that cannot be characterized clinically or pathologically as any other disease, is the most

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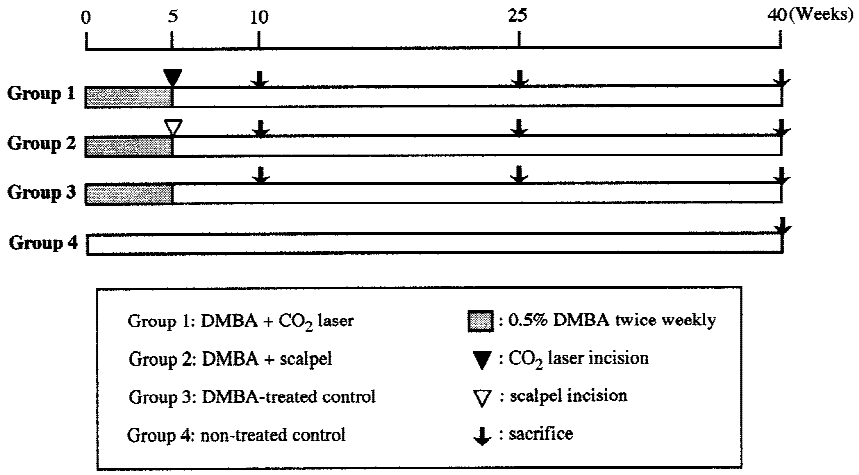

Fig. 1. Experimental design. CO_2 laser and scalpel incisions were made after 5-week applications of carcinogen. Animals were killed at weeks 10, 25, and 40 (group 4 was killed at week 40).

common and best studied premalignant lesion. The rate of malignant transformation has been reported to range from 0.13% to 36.4% [3–5]. In most cases of premalignant lesions treated by using CO_2 laser, the postoperative course has been successful, with local control rates of 90% at the 5-year follow-up [6]. Therefore, the CO_2 laser has been recognized as a useful tool for treatment of premalignant lesions. However, malignant transformation of oral leukoplakia after CO_2 laser surgery has been reported by several investigators [7–10]. The rate of malignant transformation was demonstrated to range from 2.6% to 3.0% after CO_2 laser excision and from 1.7% to 7.1% after CO_2 laser vaporization. These values were relatively higher than those after traditional scalpel surgery, which have been reported to range from 0% to 4.6% [3,5,11]. Kingsbury et al. [12] investigated the effects of surgical incisions with CO_2 laser and traditional scalpel on hamster buccal pouch epithelium initiated by carcinogen and demonstrated that the rate of malignant transformation after CO_2 laser incisions (50%) was significantly higher than that after scalpel incisions (0%). Moreover, it has been documented that the promotional effects of CO_2 laser are related to the transient increase in growth factor release [13–15]. However, the mechanism of the promotional effects of CO_2 laser on carcinogenesis has not been clarified.

The glutathione S-tranferases (GSTs) are a group of multifunctional enzymes that perform several roles in detoxication of a broad spectrum of electrophilic drugs, including carcinogens and their metabolites [16,17]. The placental form of GST (GST-P) is a useful marker of preneoplastic lesions in rat hepatocarcinogenesis [18] and hamster pancreatic carcinogenesis [19]. GST-P positivity during oral carcinogenesis has also been documented as a useful marker for premalignant and malignant lesions [20,21]. A previous experiment using 9,10-dimethyl-1,2-benzanthracene (DMBA)-induced hamster buccal pouch carcinogenesis demonstrated that GST-P was not detected in normal epithelium, that strong expression was confirmed in early premalignant lesions, particularly in the early stage, and that it decreased significantly in dysplastic lesions and especially squamous cell carcinoma [22]. The results suggested that strong expression of GST-P is a useful marker for early premalignant lesions and that secondary decrease of GST-P expression is predictive of future development of malignancy. The activity of chemical carcinogenesis is a complex balance between metabolic activation by phase I enzymes, such as cytochrome P450 monooxygenases, and detoxication by phase II enzymes, such as GSTs [23]. Recent investigations have indicated that GSTs play an important role in the detoxication of carcinogens and may prevent carcinogenesis, especially in the early stage [24,25]. It is also important to analyze cell proliferation during carcinogenesis. 5-Bromodeoxyuridine (BrdU) has been demonstrated to stain cells in the DNA synthesis phase of cell division [26]. The purpose of the present study was to investigate the kinetics of GST-P expression and cell proliferation with BrdU immunostaining after CO_2 laser surgery on the epithelium initiated by carcinogen.

MATERIALS AND METHODS

A schematic presentation of the experimental design is shown in Figure 1. A total of 94 male

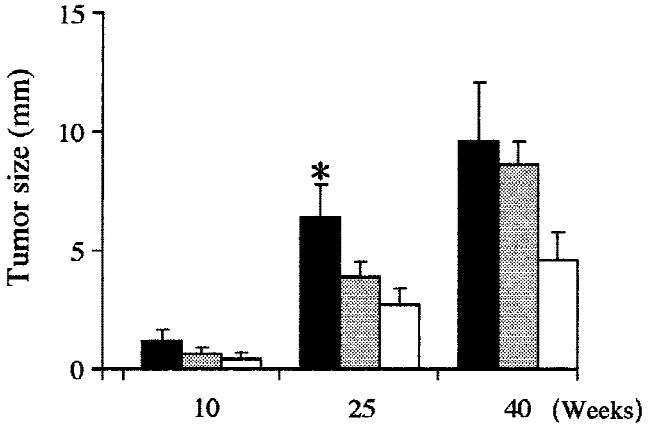

Fig. 2. Effects of treatment on tumor size. Values represent the mean ± SEM. Solid bar, group 1 [9,10-dimethyl-1,2-benzanthracene (DMBA) + CO_2 laser]; gray bar, group 2 (DMBA + scalpel); open bar, group 3 (DMBA alone). *$P < 0.05$, significantly different from DMBA alone.

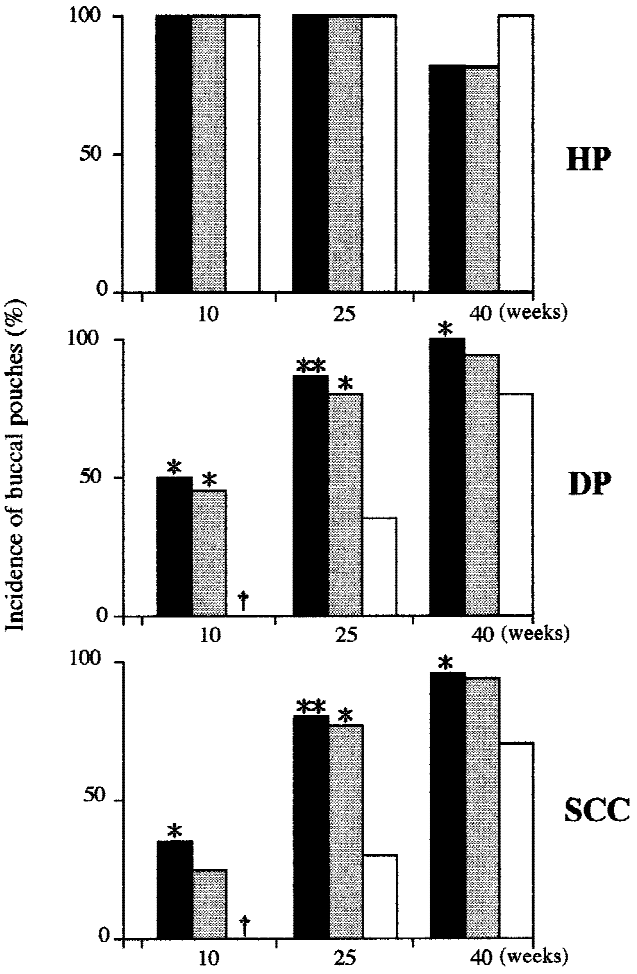

Fig. 3. Values represent the incidences of each lesion. solid bar, group 1 [9,10-dimethyl-1,2-benzanthracene (DMBA) + CO_2 laser]; gray bar, group 2 (DMBA + scalpel); open bar, group 3 (DMBA alone). HP, hyperplasia; DP, dysplasia; SCC, squamous cell carcinoma. *$P < 0.05$, **$P < 0.001$, significantly different from DMBA alone. † indicates DP or SCC was not detected in group 3 at week 10.

Syrian golden hamsters (Japan SLC Inc., Hamamatsu, Japan), five weeks old and weighing 70–80 g at the beginning of the experiment, were maintained on a basal diet (Oriental MF, Oriental Yeast Co. Ltd., Tokyo, Japan) and water ad libitum. They were housed in individual plastic cages in an air-conditioned room at 24°C. Bilateral buccal pouches of 89 animals were topically applied with 0.5% DMBA (Sigma Inc., St. Louis, MO) in mineral oil solution, 0.05 ml twice weekly for five weeks. A previous study using DMBA-induced hamster buccal pouch carcinogenesis demonstrated that five-week applications of DMBA induced only hyperplastic lesions in all buccal pouch epithelium at week 5, and the rates of malignant transformation were 30% and 70% at weeks 25 and 40, respectively [22]. Based on these results, hyperplastic lesions induced by five-week applications of DMBA can be regarded as a suitable model of premalignant lesions. After DMBA application (at the beginning of week 6), CO_2 laser incisions were made bilaterally on the buccal pouch epithelium of 36 animals (group 1, G1), and scalpel incisions were similarly made on 33 animals (group 2, G2). Buccal pouches of 20 animals not treated further were used as the DMBA-treated controls (group 3, G3). Five animals served as nontreated controls (group 4, G4). A Luxar CO_2 laser (LX-20, Luxar Co., Seattle, WA; energy beam wavelength = 10,600 nm; spot size = 0.4 mm in diameter) was used with a handpiece. A power setting of 2 W in continuous mode was selected. All incisions were made over the carcinogen-initiated region, like a grid, over as wide an area as possible, and 0.5 mm in depth. At that time, no tumorous lesions were detected in any buccal pouch epithelium. Animals were killed at week 10 (G1, G2: 10 animals; G3: five animals), week 25 (G1, G2: 15 animals; G3: 10 animals), and week 40 (G1: 11 animals; G2: eight animals; G3, G4: five animals). One hour before being killed under ether anesthesia, all animals were injected intraperitoneally with BrdU (Sigma) at a dose of 75 mg/kg body weight. Bilateral buccal pouches were excised and observed grossly. Sequentially removed specimens were fixed in cold acetone, processed routinely, and embedded in paraffin. Three serial sections from individual pouches were cut and mounted on glass slides. One slide was stained with hematoxylin and eosin

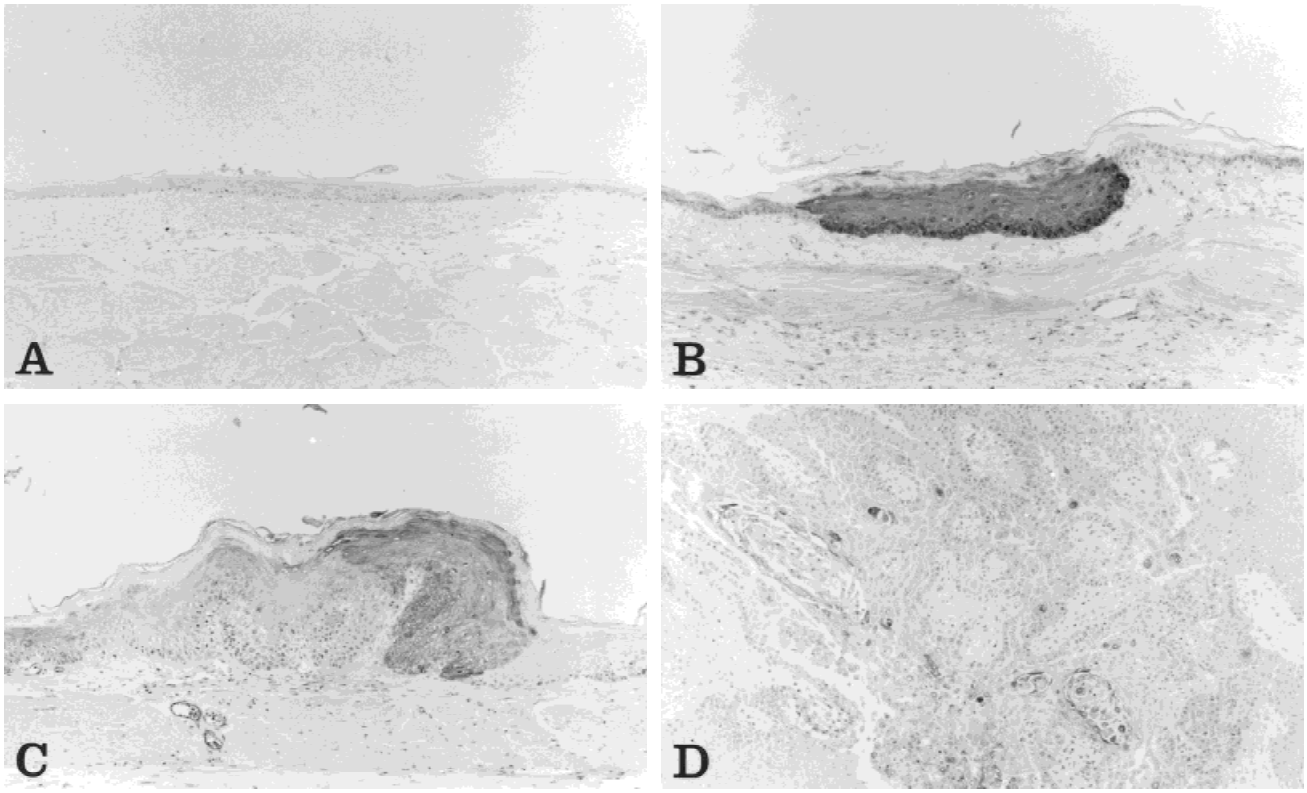

Fig. 4. Photomicrographs showing expression of glutathione S-transferase placental form (GST-P) in normal epithelium and for each lesion. **A:** Normal epithelium obtained from nontreated control animal show no positivity. Expression was strong in hyperplastic lesions (**B**), moderate in dysplastic lesions, and weak in squamous cell carcinoma (**D**). GST-P immunostain, ×100.

(HE) for histopathologic observation. The other two slides were used for GST-P immunohistochemistory and cell proliferation assays.

Gross Observation

The sizes (mm) of the tumors, which were taken to be the mean of the long and short lengths, were grossly determined in each group. When no tumor formation was observed, the tumor size was detemined to be 0 mm. When more than two tumors were found in a pouch, the larger one was measured.

Histopathologic Observations

Using HE-stained sections, lesions in the buccal pouch epithelium were classified histopathologically into three categories: hyperplasia (HP), dysplasia (DP), and squamous cell carcinoma (SCC). The incidences of each lesion were determined.

GST-P Immunohistochemical Analysis

Immunohistochemical staining using the avidin-biotin-peroxidase complex (ABC) method (Vectastain Elite ABC kit, Funakoshi Inc., Tokyo, Japan) was used to determine the expression of GST-P in individual lesions. Paraffin sections were routinely passed through xylene and a graded alcohol series and then treated sequentially with normal goat serum, rabbit anti-rat liver GST-P antibody (1:16,000, MBL Inc., Nagoya, Japan), biotin-labeled goat anti-rabbit IgG, and ABC. The sites of peroxidase binding were detected with a solution of 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB), and the sections were counterstained with hematoxylin. Sections were used to determine the percentages of GST-P-positive areas for individual lesions by using a color image processor (SPICCA, Nippon Avionics Co., Tokyo, Japan), and the mean values of the three lesions classified histopathologically were calculated.

GST-P-positive area = (GST-P positive area/whole area of individual lesion) × 100.

Hepatic tissues were used as a positive control.

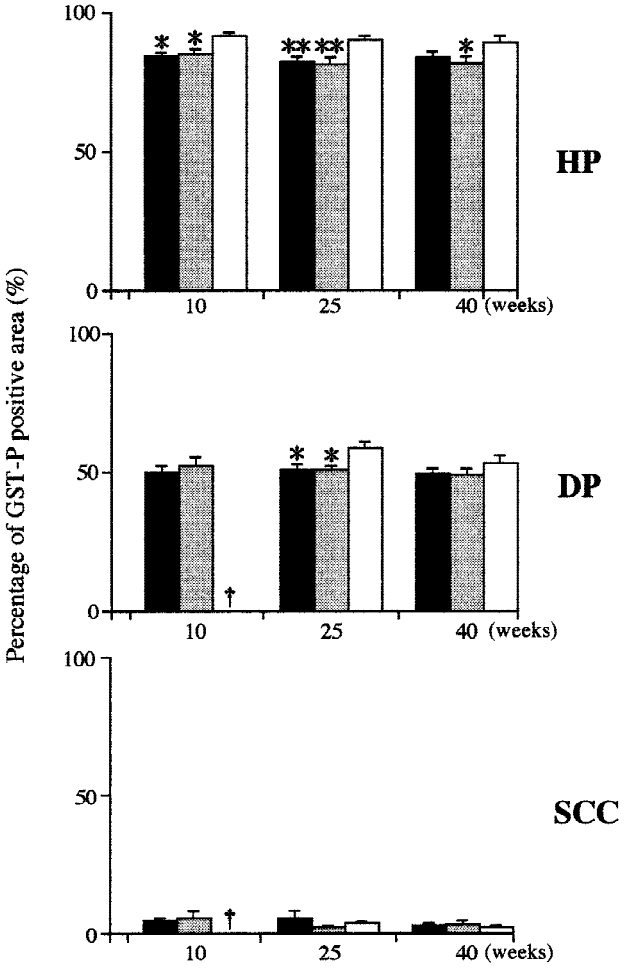

Fig. 5. Percentages of areas positive for the placental form of glutathione S-transferase (GST-P) in each lesion. Values represent the mean ± SEM. Solid bar, group 1 [9,10-dimethyl-1,2-benzanthracene (DMBA) + CO_2 laser]; gray bar, group 2 (DMBA + scalpel); open bar, group 3 (DMBA alone). HP, hyperplasia; DP, dysplasia; SCC, squamous cell carcinoma. *$P < 0.05$, **$P < 0.001$, significantly different from DMBA alone. † indicates DP or SCC was not detected in group 3 at week 10.

Cell Proliferation Assays

BrdU immunohistochemical staining was achieved by using the ABC method. The sections were deparaffinized and treated sequentially with 1 N HCl, mouse anti-BrdU monoclonal antibody (1:1,000, DAKO Inc., Santa Barbara, CA), biotin-labeled horse anti-mouse IgG, ABC, a solution of 0.05% DAB, and counterstaining with hematoxylin. Numbers of BrdU-labeled cells per 2,000 cells in individual lesions were counted by light microscopy, and BrdU labeling indices (BrdU LIs) were calculated as percentage values:

BrdU LI = (number of BrdU-labeled cells/total number of cells in the lesion) × 100.

In HP, BrdU LIs for the GST-P-positive and -negative areas were calculated separately. Intestinal tissues were employed as a positive control.

Statistical Analysis

The chi-square test was used to analyze the incidence of each category of lesion classified histologically, and tumor size, GST-P immunohistochemistry, and cell proliferation were evaluated by Student's t-test. A level of $P < 0.05$ was considered statistically significant.

RESULTS

Gross Findings

The tumor sizes of each group determined grossly are illustrated in Figure 2. In the DMBA-treated control group, permissive growth of tumor was found in proportion to experimental time, but the tumor sizes of two experimental groups, particularly in G1, indicated remarkable growth. At

TABLE 1. BrdU LI of Each Lesion*

		BrdU LI (%)		
	(W)	HP	DP	SCC
Group 1.	10	2.67 ± 0.16	3.58 ± 0.24	8.34 ± 0.88
(DMBA + CO_2 laser)	25	2.47 ± 0.15	3.78 ± 0.18	7.79 ± 0.85
	40	2.46 ± 0.10	4.20 ± 0.20	8.37 ± 0.72
Group 2.	10	2.51 ± 0.16	3.44 ± 0.21	9.64 ± 0.75
(DMBA + scalpel)	25	2.65 ± 0.14	3.73 ± 0.15	7.89 ± 0.58
	40	2.28 ± 0.11	4.05 ± 0.27	8.74 ± 0.60
Group 3.	10	2.34 ± 0.26	—	—
(DMBA alone)	25	2.42 ± 0.17	3.66 ± 0.92	8.20 ± 0.54
	40	2.35 ± 0.22	3.63 ± 0.28	8.77 ± 1.39

*Values represent mean ± SEM.

week 25, the value of G1 was significantly ($P < 0.05$) greater than that of the DMBA-treated control group. In the nontreated control group, no change in epithelium was detected.

Histopathologic Findings

Figure 3 shows the incidence of each category of lesion classified histopathologically. In the DMBA-treated control animals, HPs were found in all buccal pouches. In G1 and G2, HPs were similarly detected in all pouches until week 25, but the incidence decreased at week 40. In the DMBA-treated control group, DPs and SCCs were observed from week 25, and the incidence of these lesions increased gradually. Conversely, in G2 and especially in G1, DPs and SCCs appeared from week 10, and incidence increased rapidly. The incidence of SCCs and DPs in G1 was significantly higher than that in the DMBA-treated control group at all death times (weeks 10 and 40: $P < 0.05$; week 25: $P < 0.001$). At week 25, the incidence of SCCs and DPs in G2 was also significantly ($P < 0.05$) higher than that of the DMBA-treated control group. However, the incidence for these three categories of lesion failed to show any significant difference between G1 and G2. In the nontreated control animals, no lesions were detected.

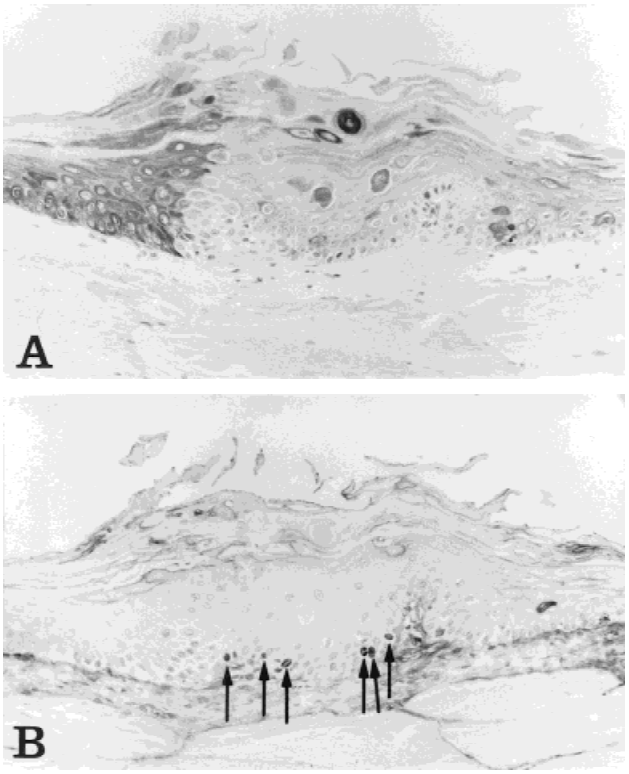

Fig. 6. **A:** Photomicrograph showing decreased expression of the placental form of glutathione S-transferase (GST-P) in hyperplasia after CO_2 laser incisions. **B:** The number of 5-bromodeoxyuridine-labeled cells (arrows) is greater in the GST-P-negative area than in the GST-P-positive area. ×200.

Expression of GST-P

Percentages of GST-P-positive areas in each of the three categories of lesions are shown in Figures 4 and 5. Strong expression was confirmed in HP, followed by DP, and very little expression was found in SCC. HPs observed in the DMBA-treated control group, which had a potential for slow progress of carcinogenesis, indicated the highest percentage of GST-P-positive area. Conversely, in HPs of both G1 and G2, the percentages GST-P-positive areas were significantly lower than those in the DMBA-treated control group (G1 at week 10, G2 at weeks 10 and 40: $P < 0.05$; G1 at week 25 and G2 at week 25: $P < 0.001$). Also, in DPs, significantly lower percentages of GST-P-positive areas compared with those of the DMBA-treated control group were confirmed in both G1 and G2 at week 25 ($P < 0.05$). However, in SCCs, GST-P-positive areas were small, and the percentages were not influenced by surgical treatment. In the nontreated animals, no GST-P positivity was detected in normal epithelium. Moreover, normal-looking epithelium of all treated animals indicated no positivity of GST-P.

Cell Proliferation as Assessed in Terms of BrdU LI

The BrdU LIs of each lesion category are presented in Table 1. The increase of BrdU LIs occurred with the progression of carcinogenesis. The BrdU LIs for HPs in G1 and G2 tended to be higher than those in the DMBA-treated control group, but the BrdU LIs for each category failed to show any significant difference between groups. However, in the HPs of each of the three groups, the BrdU LIs of GST-P-negative areas were found to be higher than those for the GST-P-positive areas in the same group. In G1 at all times of death, in G2 at weeks 10 and 25, and in the DMBA-treated control group at week 25, significant (G1 at all times of death and DMBA-treated control group at week 25: $P < 0.05$; G2 at weeks 10 and 25: $P < 0.001$) differences were confirmed between BrdU LIs of the GST-P-negative areas and the GST-P-positive areas (Figs. 6, 7).

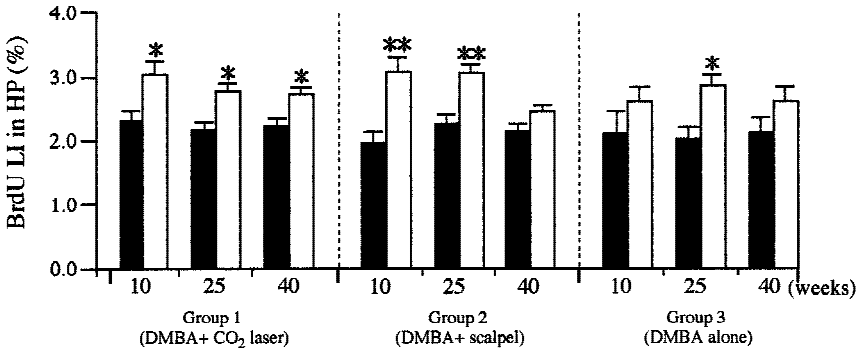

Fig. 7. 5-Bromodeoxyuridine labeling indices (BrdU LIs) of areas negative and positive for the placental form of glutathione S-transferase (GST-P) in hyperplasia. Values represent the mean ± SEM. Solid bar, GST-P-positive area; open bar, GST-P-negative area. $*P < 0.05$, $**P < 0.001$, significantly different from GST-P-positive area.

DISCUSSION

The present gross and histopathologic findings demonstrate that the malignant transformation was significantly promoted by both types of incisions. CO_2 laser incisions resulted in the promotion of carcinogenesis, as seen in the previous report [12], but no significant difference was found in the present study. Crean et al. [13], Liebow et al. [14], and Kozacko et al. [15] reported that the increase of growth factor release after CO_2 laser surgery on the initiated area promoted carcinogenesis on the hamster buccal pouch epithelium. Yu et al. [27] explored the expression of growth factors in early wound healing in rat normal skin and demonstrated that the strongest expression of growth factors was confirmed at 24 hr after the incisions both by CO_2 laser and scalpel, but there was no significant difference between these two instruments. Therefore, it was considered that the promotional effects of CO_2 laser incisions related to the increase in growth factors may not be continuous. In the present study, the percentages of GST-P-positive areas in HPs of both experimental groups (G1 and G2) showed a significant decrease when compared with those of DMBA-treated control group. Also, in HPs, the BrdU LIs of the GST-P-negative areas were significantly higher than those for the GST-P-positive areas. Our preliminary study on cell proliferation after incisions of both CO_2 laser and scalpel on the normal buccal pouch epithelium confirmed that cell proliferation had finished by five weeks after surgery.

GST-P has been demonstrated to be resistant to the antiproliferation effect of TGFβ [28]. Expression of TGFβ during DMBA-induced hamster buccal pouch carcinogenesis has been documented to increase in HP lesions but to decrease in DP lesions [29]. Our previous study demonstrated strong expression of GST-P and lower cell proliferation in HP lesions but a decrease in GST-P expression and an increase in cell proliferation in DP lesions [22]. Also, in HP lesions, the BrdU LIs of the GST-P-negative areas were confirmed to be higher than those for the GST-P-positive areas. Based on these findings, it was supposed that in HP GST-P-positive areas increased so as to counteract the antiproliferation effect of TGFβ, whereas in DP both decline in tandem.

Henderson et al. [24] investigated the function of GST-π during skin tumorigenesis by using GST-π null mice and demonstrated a highly significant increase of tumor incidence in the GST-π null group versus the GST-π (+) control group. They concluded that GST-π may be an important determinant in cancer development. Our results support the previous working hypothesis that GSTs may prevent cancer development [25]. However, the underlying mechanisms require additional study. In conclusion, our results suggest that the incisions, particularly by CO_2 laser, on the initiated areas made GST-P expression decrease and cell proliferation in the GST-P-negative areas increase. These incisions may contribute to malignant transformation.

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